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New discovery of the freshwater sponge *Ephydatia fluviatilis* (Linnaeus, 1759) in cenote ecosystems of Yucatan, Mexico: Morphological and molecular insights

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Abstract

For over five decades, the biodiversity of freshwater sponges from Yucatan cenotes has remained largely unexplored. Scientific documentation is limited to studies from 1936 and 1968, which highlights a significant knowledge gap. It is here reported the presence of the freshwater sponge *Ephydatia fluviatilis* in the Sabak-Ha and Suhem cenotes of Yucatan, Mexico. It is provided detailed descriptions of the spicules to accurately characterize morphology and ease comparison with other *Ephydatia* species. Additionally, we conducted molecular analyses using DNA sequences from the ITS1, 5.8S, and ITS2 regions, as well as partial 18S and 28S sequences, to determine phylogenetic relationships within the Spongillidae family. DNA sequences from both cenotes were nearly identical to *E. fluviatilis* GenBank sequences (<3% divergence), confirming species identification. Spicule analysis revealed gemmuloscleres birotules with shafts densely covered by spines as long as the rotular rays as recorded throughout the entire geographic range of this species in both lentic and lotic water bodies. This study expands our knowledge of the diversity and distribution of freshwater sponges in Yucatan cenotes, where only *Spongilla cenota* and

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Radiospongilla crateriformis had been previously reported. Our results lay the groundwork for future research on cenote sponge biodiversity in this unique karst ecosystem and underscore the importance of integrating molecular and morphological data for accurate species identification.

Keywords
Biodiversity, Karst environment, Porifera, Sabak-Ha Cenote, Sinkholes, Spongillida, Spongillidae, Suhem Cenote, Yucatan Peninsula

Introduction

Cenotes are aquatic ecosystems with characteristic karst formations located throughout the Yucatan Peninsula, they represent Mexico's hydrogeological heritage (Beddows et al. 2007). These systems originate from the dissolution of limestone and the subsequent collapse of underground cavities' roofs. These cavities are fed by rainfall infiltration through calcareous soil structures, forming circular depressions connected to aquifers (Schmitter-Soto et al. 2002). The interaction between fresh and marine water generates chemical and biological stratification in these environments, as a result, cenotes constitute biodiversity reservoirs with high levels of endemism. These formations function as main connectors to the aquifer, housing highly specialized biological communities such as stygobiont crustaceans and fish adapted to low-luminosity and low-oxygen conditions (Camargo-Guerra et al. 2013; Álvarez et al. 2015; Angyal et al. 2021). This environment provides invaluable information about the dynamics of groundwater and the biogeochemical processes occurring in these ecosystems (Socki et al. 2002).

Recent studies of aquatic biodiversity in cenotes main focus is on crustacean taxa, including Copepoda, Remipedia, Ostracoda, Thermosbaenacea, Mysida, Amphipoda, Isopoda, and Decapoda (Suárez-Morales and Reid 2003; Boxshall et al. 2014; Álvarez et al. 2015; Angyal et al. 2018, 2020a, 2020b; Chávez-Solís et al. 2018; Espinasa et al. 2019; Chávez-Solís et al. 2020; Liévano-Beltrán and Simões 2021; Macario-González et al. 2021; Jaime et al. 2025), also fish from the families Cichlidae, Poeciliidae, Characidae, Pimelodidae, and Synbranchidae (Medina-González et al. 2001; Camargo-Guerra et al. 2013; Schmitter-Soto 2020), as well as gastropod mollusks (Grego et al. 2019) and photosynthetic microorganisms, particularly cyanobacteria and phytoplankton have been studied (Águila et al. 2022; Díaz-Hernández et al. 2023). However, for other taxa, such as sponges (Phylum Porifera), there has been a significant gap in research regarding the updating of their species richness, ecology and distribution in cenotes.

The first sponge species documented in cenotes from Yucatan were *Spongilla lacustris* (Linnaeus, 1759) and *Ephydatia crateriformis*, which is now recognized as *Radiospongilla crateriformis* (Potts, 1882), both were identified by Old (1936b). Penney and Racek (1968) later described a new species, *Spongilla cenota*, from a different cenote than those studied by Old (Table 2). Subsequent research by Poirrier (1976) confirmed

Table 1. Spicular morphometry of *Ephydatia fluviatilis* specimens by cenote. Measurements are presented as mean (minimum-maximum) values for oxeas length × width. For birotules gemmuloscleres, measurements include length × shaft width × rotule diameter.

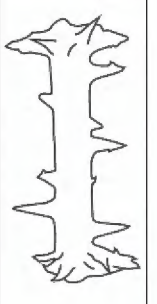
Cenote	Specimen	Smooth oxeas I (μm)	Smooth oxeas II (μm)	Spiny oxeas (μm)	Birotules (μm)
Sabak-Ha	CNPGG-2670 (Fig. 3C)	316.11 (274.74–359.11) × 15.9 (14.51–17.66)	184.18 (168.67–199.68) × 5.04 (4.93–5.14)	110.71 (88.92–132.51) × 10.13 (9.1–11.17)	53.32 (50.47–55.39) × 8.67 (6.81–10.15) × 24.43 (21.43–31.74)
Sabak-Ha	CNPGG-2671 (Fig. 3D)	335.07 (309.89–365.42) × 18.17 (15.97–19.71)	172.2 (167.94–176.45) × 4.8 (4.67–4.93)	Not found	57.93 (44.65–72.96) × 8.31 (5.24–9.9) × 22.75 (21.33–23.54)
Suhem	CNPGG-2673 (Fig. 5C)	328.13 (243.28–373.53) × 13.19 (10.09–15.85)	161.29 (147.27–175.3) × 4.25 (3.77–4.73)	Not found	37.74 (32.66–43.15) × 5.61 (4.64–6.96) × 22.92 (19.18–26.18)

that the material previously identified as *S. lacustris* in cenotes was actually *S. cenota*. Consequently, all specimens of *S. lacustris* found in Yucatan Peninsula cenotes were reassigned to *S. cenota*. Since these early studies, the biodiversity of freshwater sponges in Yucatan Peninsula cenotes has remained largely unexplored.

Mexico spans both the Nearctic and Neotropical regions, there, approximately 10 species of freshwater sponges (Spongillidae) have been documented across various aquatic ecosystems, including lakes, lagoons, and rivers in the country's central and northern areas (Ehrenberg 1841; Potts 1885a, 1885b; Arndt 1933; Zorrilla 1935; Old 1936a; Martínez 1940; Rioja 1940a, 1940b, 1940c; Rioja and Herrera 1952; Rioja 1953; Bushnell 1971; Manconi and Pronzato 2005; Carballo et al. 2017, 2021; Gómez et al. 2019). In contrast, there are only two reports of marine sponges in cenotes from Quintana Roo, specifically those located near the coast with an influx of seawater, one from the genus *Xestospongia* (Suárez-Moo et al. 2024), and the marine endemic species *Amphibleptula aaktun* from the anchialine Aerolito cenote (Gómez et al. 2021). Notably, these marine sponges do not belong to the order Spongillida, which comprises predominantly freshwater species. Additionally, an unidentified sponge was recorded in Los Sabinos Cave in San Luis Potosí in the northern part of Mexico (Legendre et al. 2023). Overall, the knowledge of freshwater sponges in Mexican subterranean ecosystems is extremely limited compared to other freshwater habitats, where they have been studied more extensively.

The genus *Ephydatia* Lamouroux, 1816, is included among the freshwater sponges reported in Mexico. There are documented old records of *E. fluviatilis* (Linnaeus, 1759) and *Ephydatia robusta* (Potts, 1888) in lacustrine ecosystems, such as Lakes Xochimilco and Pátzcuaro in central Mexico (Potts 1885b; Zorrilla 1935; Old 1936a; Penney and Racek 1968). However, there are no recent status updates of this genus in Mexico. *Ephydatia* is a cosmopolitan genus widely distributed throughout the Northern Hemisphere and present in the Southern Hemisphere as well (Manconi and Pronzato 2008; Manconi and Pronzato 2016; Rasbold et al. 2023). These sponges primarily colonize rocky substrates and tree trunks in the shallow waters of ponds, rivers, lakes, lagoons and still bodies of water (lentic habitats) (Muricy et al. 2011; Nicacio and Pinheiro

Table 2. Description, location and spicular diversity of sponges reported in Yucatan Cenotes, Mexico. Records and descriptions obtained from: [1]Old (1936b), [2]Penney and Racek (1968). The spicules are presented with average length measurements. Spicule drawings for *Spongilla cenota* and *Radiospongilla crateriformis* were made based on the illustrations descriptions by Old (1936b). Drawings depicting *Ephydatia fluviatilis* were rendered directly from specimens examined in the current study.

Species	External description	Cenote registration	Spicules		
			Oxeas	Birotules	Microscleres
Radiospongilla crateriformis (Potts, 1882)	Sponge encrusting, thin, texture very loose, forming no tangible skeleton.[1]	**Dzadz Aguada**[1] (10 km southwest of Chichen Itzá), Yucatán, México	260 µm	90 µm	
Spongilla cenota (Penney & Racek, 1968)	Sponge irregular, and more or less massive cushions, surface smooth.[2]	**Xtoloc Cenote**[2] (20.68°N, -88.56°W), **Xanaba Cenote Grande**[1] (8 km southwest of Chichen Itzá), **Xanaba Cenote**[1] (8 km southeast of Chichen Itzá), **Dzadz Aguada**[1], Yucatán, México	310–410 µm	65–86 µm	68–123 µm
Ephydatia fluviatilis (Linnaeus, 1759)	Sponge massive, encrusting, often forming a discoidal growth.	**Sabak-Ha Cenote** (20.58°N, -89.58°W). **Suhem Cenote** (20.64°N, -89.40°W), Yucatán, México	147–365 µm	44–72 µm 	

2015; Evans and Montagnes 2019; Gost et al. 2023). There, they play important ecological roles in water filtration, food webs, and symbiotic interactions (Hall et al. 2021; Saxena 2021; Giudice and Rizzo 2024). Their extensive distribution and remarkable ability to thrive in diverse habitats make them particularly valuable for evolutionary and paleolimnological studies (Harrison 1988; Manconi and Pronzato 2016; Pisera et al. 2016). Considering the lack of updated information on *Ephydatia* in Mexican ecosystems, despite its ecological significance and scientific value, it is crucial to conduct new surveys to document its current distribution patterns.

This study documents the presence of *Ephydatia fluviatilis* in two cenotes in Yucatán, Mexico, using morphological by both Light Microscopy (LM) and Scanning Electron Microscopy (SEM) and molecular analyses to clarify its taxonomic identity and systematic position, including the investigation of diagnostic microcharacters. Our findings represent the first record of this species in subterranean freshwater ecosystems of the region and the first update for Mexico in over 80 years since its documentation in Lake Xochimilco (Rioja 1940a). This research expands current knowledge of sponge biodiversity in cenote environments, updates the known distribution of *E. fluviatilis*, and highlights its capacity to colonize diverse habitats. Furthermore, it provides a foundation for future studies on the ecology and biogeography of freshwater sponges in cenote ecosystems.

Materials and methods

Sampling sites

Sabak-Ha Cenote is located at the municipality of Sacalum in Yucatan, Mexico (20.5801°N, 89.5881°W), while Suhem Cenote is located at the municipality of Pixya in Yucatan, Mexico (20.6403°N, 89.4031°W) (Fig. 1). Both are open-type cenotes (Figs 2A, 4A).

Sabak-Ha is 60 m long and 30 m wide, with a recorded depth of 120 m, although its true depth is unknown (SDS 2024). From the surface to an average depth of 15 m, Sabak-Ha has a layer of organic matter with extremely low visibility where the sponge has been found attached to its walls, while no sponges have been found in the crystal-clear waters below this layer. The depth of Suhem ranges from 4 m to around 35 m at its deepest point and is a popular site for tourist activities (Cenoteando 2025).

Sampling and identification

Sponges were collected in both cenotes by SCUBA (Self-Contained Underwater Breathing Apparatus) and the specimens were placed into the national collection "Colección Nacional del Phylum Porifera – Gerardo Green" (**CNPGG**) at the Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México (**UNAM**). In Sabak-Ha, four samples from four individual sponges were collected at

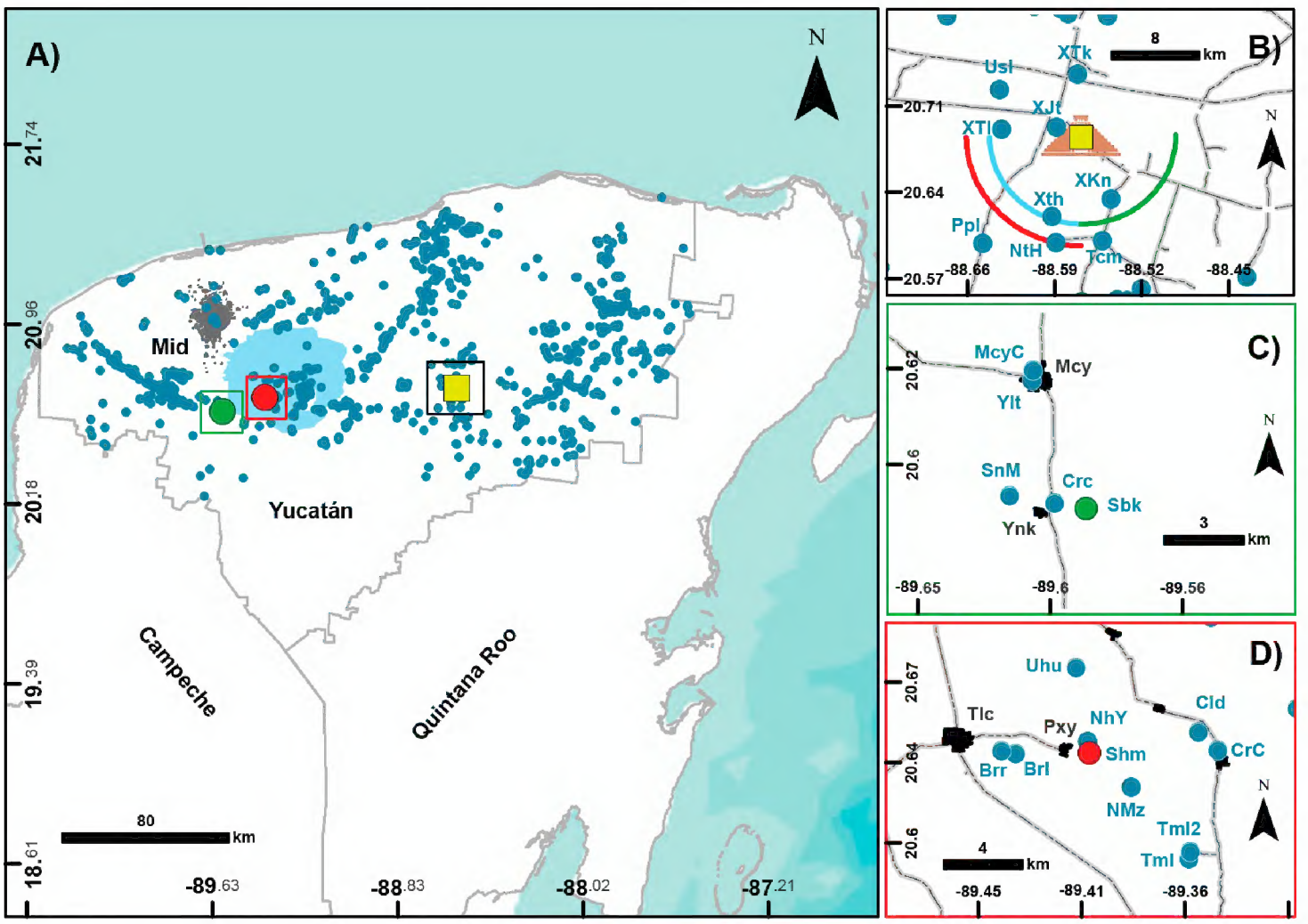


Figure 1. Records of *Ephydatia fluviatilis* in cenote ecosystems of Yucatan, Mexico. **A** records in Yucatan Cenotes: Sabak-Ha (green point) and Suhem (red point). The record of *Spongilla cenota* at Xtoloc Cenote is also shown (yellow square) (Penney and Racek 1968). Blue points indicate cenote locations. Gray area represents Mérida city (Mid), the capital city of Yucatan. The sky-blue area represents the protected natural area "Reserva Estatal Geohidrológica Anillo de Cenotes" (SDS 2013) **B** close-up of the Chichen Itzá area (brown triangle) illustrating the potential locations of cenotes where Old (1936b) documented *Radiospongilla crateriformis* and *S. cenota*. The red line indicates a limit of 10 km southwest of Chichen Itzá, where *R. crateriformis* was recorded in the Dzadz Aguada. Light blue and green lines represent areas 8 km southwest and southeast, respectively of Chichen Itzá, where *S. cenota* was documented in Xanaba Cenote (southeast) and Xanaba Cenote Grande (southwest). Surrounding cenotes are also displayed: X'Tikinka (XTk), Usil (Usl), X'Jotun (XJt), X'Tojil (XTl), X'Katún (XKn), X'Tohil (XTh), Ticimul (Tcm), Nicte-Ha (NtH) and Popola (Ppl) **C** a close-up view of the area surrounding Sabak-Ha Cenote (Sbk). Nearby settlements (black areas) are labeled in black: Mucuyché (Mcy) and Yunkú (Ynk), with regional cenotes designated in blue: Mucuyché (McyC), Yaal Utzil (Ylt), San Marco (SnM), Crucero (Crc). The gray line depicts the local road network **D** a close-up view of the area surrounding Suhem Cenote (Shm). Nearby settlements (black areas) are labeled in black: Telchaquillo (Tlc) and Pixya (Pxy), with regional cenotes designated in blue: Uhua (Uhu), Burro (Brr), Burro I (BrI), Caldero (Cld), Nah-Yah (NhY), Cruz Chen (CrC), Noh-Mozón (NMz), Ti'Muul (Tml) and Ti'Muul 2 (Tml2). The gray line depicts the local road network.

depths of 4 m, 6 m, 8 m and 11 m (Fig. 3). All samples were analyzed using Light Microscopy (LM). Two specimens, corresponding to the light (CNPGG-2670) and dark (CNPGG-2671) morphotypes, were deposited in the CNPGG and were used for spicule measurements and Scanning Electron Microscopy (SEM) imaging. Three samples were submitted for molecular sequencing, although one failed to yield usable

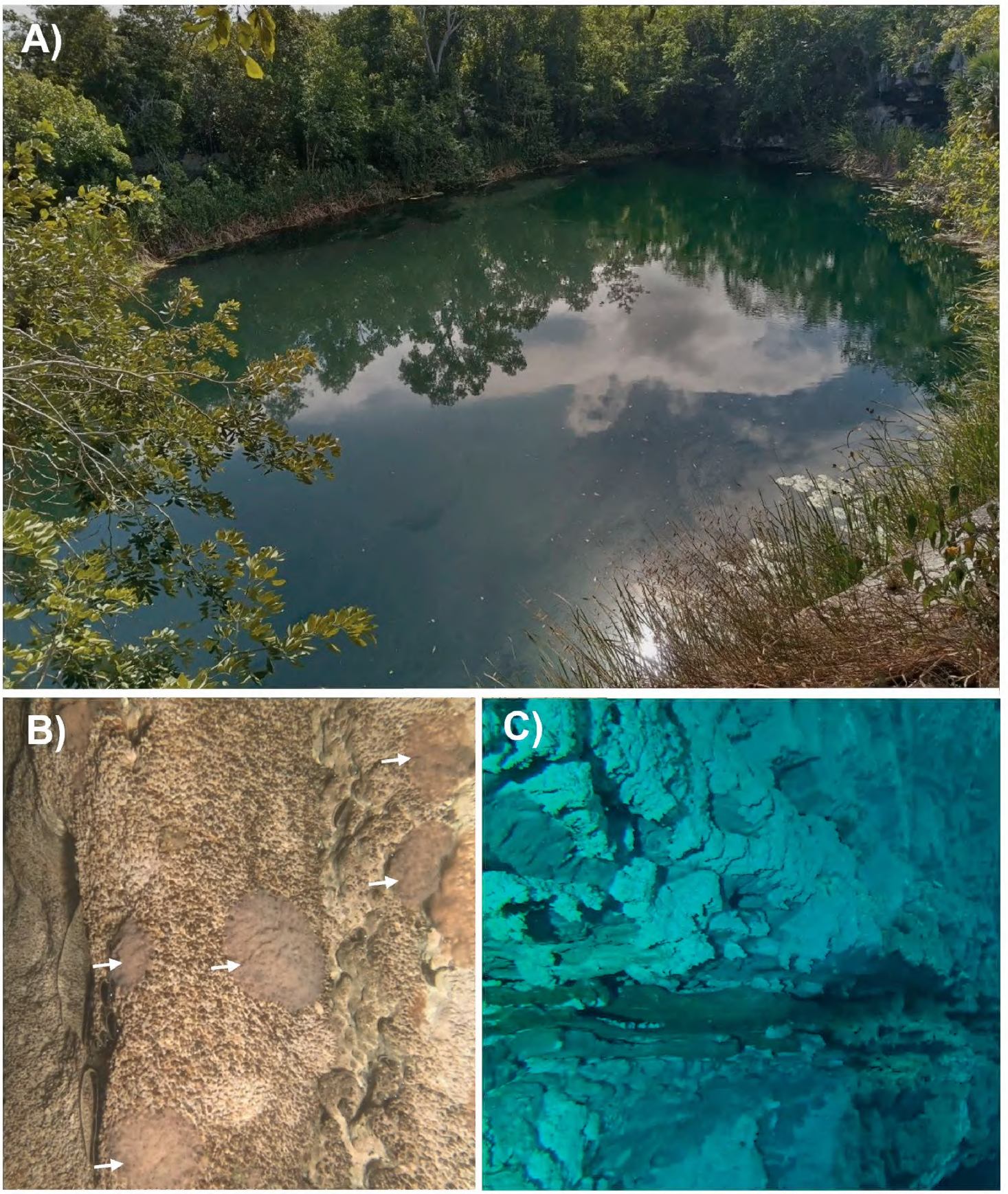


Figure 2. Cenote Sabak-Ha in Sacalum, Yucatan. **A** external view of the cenote **B** general view of cenote wall at 11 m depth in the organic matter layer where the sponges (indicated with arrows) were found **C** general view of cenote wall in clear water zone at 35 m depth.

results. In Suhem, samples from three individual sponges were collected at depths of 4 m, 4.7 m and 5.3 m (Fig. 5). All samples were analyzed by LM; due to their morphological similarity, only one specimen (CNPGG-2673) was deposited in the CNPGG and selected for molecular sequencing. All samples were fixed and preserved in alcohol (96%). Sponges were photographed *in situ* (Figs 3, 5).

Small sponge fragments were manually cut and prepared for detailed microscopic analysis and examination of the spicules arrangement within the skeletal architecture. The procedure for the Scanning Electron Microscopy (SEM) observation followed the methodological standards established by Hooper (2003). Spicules and gemmules were cleaned with nitric acid and exhaustively washed with distilled water to remove organic material and allow detailed examination. Sponge fragments were hand-sectioned for the analysis of spicule arrangement within the skeletal architecture. For Light Microscopy (LM), cleaned spicules and gemmules were mounted on glass slides using Canada balsam to ensure permanent preservation, whereas skeletal sections were embedded in Entellan® resin. For scanning electron microscopy (SEM), cleaned spicules were mounted on aluminum stubs, and coated with gold. Spicule dimensions were determined by measuring fifteen representative spicules from each morphological category, and results were reported as average length and width, including minimum and maximum values.

The morphological identification of the sponges followed Manconi and Pronzato (2002) for genus-level classification, and the descriptions of Potts (1888) and Penney and Racek (1968) for species-level identification. The systematic classification adheres to the authoritative World Porifera Database (de Voogd et al. 2024).

Molecular analysis

DNA was extracted from 96% ethanol-preserved sponge samples collected in this study, two from Cenote Sabak-Ha (samples taken at two different times), and one sample from Cenote Suhem, the extraction was performed using the Qiagen DNeasy blood and Tissue Kit.

For amplification primers were selected according to sequences of freshwater sponges from GenBank, and studies have shown that it has a higher variability in all demosponge subclasses. The primers used were RA2 (5'- GTC CCT GCC CTT TGT ACA CA -3') and ITS2.2 (5'- CCT GGT TAG TTT CTT TTC CTC CGC – 3'), the amplified fragments would thus include ITS1, 5.8S, ITS2 and small parts of the 18S and 28S regions (Wörheide 1998). The cycle parameters were initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension of 5 min at 72 °C. PCR-products were run in a 1.2% agarose gel to corroborate the positive amplification. The sequences of cenote sponges obtained in this study are deposited in GenBank (http://www.ncbi.nlm.nih.gov) with the accession numbers PX492551, PX492552 and PX492553.

The obtained sequences were assembled and edited with Geneious Prime 2025.2.2 (https://www.geneious.com). The assignment of the sequences obtained was performed using the BLAST check on NCBI GenBank (http://www.ncbi.nlm.nih.gov/blast/). Sequences were initially aligned using Muscle on Geneious under default parameters and adjusted manually, including some available sequences of related freshwater sponges available from GenBank, resulting in a data matrix of 21 samples and 813 bp, including

sequences of cenote sponge and members of the family Spongillidae. Based on the latest studies, we used *Spongilla lacustris* EF151944, *Radiospongilla* (Penney & Racek, 1968) EF151952 and *Trochospongilla* (Veidoyský, 1883) PP853641 as outgroups (Itskovich et al. 2008, 2015; Itskovich et al. 2017, 2022; Gómez et al. 2019; Zhao et al. 2023).

Phylogenetic analyses were performed by Maximum Likelihood (ML) using PhyML (Guindon et al. 2010) and Bayesian Inference (BI) using MrBayes 3.2 (Ronquist et al. 2012). For the ML analysis, the GTR+G+I model was selected as the best-fitting model. Node support was estimated by bootstrap resampling with 500 replicates (Felsenstein 1985). Bayesian analyses were run with four Markov chains for 1,100,000 generations, sampling every 200 generations, using the GTR+G+I model selected by JModelTest 2.1.10. program (Darriba et al. 2012). The first 25% of sampled trees were discarded as burn-in. Convergence was assessed by examining the average standard deviation of split frequencies, which fell below 0.01. Additionally, parameter stationarity was verified using Tracer v1.7, ensuring that Effective Sample Size values for all parameters exceeded 200.

Results

Systematics

Phylum Porifera Grant, 1836
Class Demospongiae Sollas, 1885
Subclass Heteroscleromorpha Cárdenas, Pérez & Boury-Esnault, 2012
Order Spongillida Manconi & Pronzato, 2002
Family Spongillidae Gray, 1867
Genus *Ephydatia* Lamouroux, 1816

***Ephydatia fluviatilis* (Linnaeus, 1759)**
Figs 3,5

Synonymy and references. *Clypeatula cooperensis* Peterson & Addis, 2000, *Ephydatia fluviatilis chui* Gee, 1926, *Ephydatia fluviatilis hastifera* Rezvoj, 1930, *Ephydatia fluviatilis intha* Annandale, 1918, *Ephydatia fluviatilis teberdana* Rezvoj, 1928, *Ephydatia fluviatilis* var. *capensis* Kirkpatrick, 1907, *Ephydatia fluviatilis* var. *himalayensis* Annandale, 1912, *Ephydatia goriaevii* Swartschewsky, 1901, *Meyenia fluviatilis* (Linnaeus, 1759), *Meyenia fluviatilis* var. *angustibirotulata* Carter, 1885, *Meyenia fluviatilis* var. *gracilis* Carter, 1885, *Meyenia mexicana* Potts, 1885, *Spongilla fluviatilis* Linnaeus, 1759, *Spongilla fluviatilis* (Linnaeus, 1759), *Spongilla pulvinata* Lamarck, 1816, *Spongilla sceptrifera* Bowerbank, 1874, *Spongilla stagnalis* Dawson, 1875.

Type locality. The Netherlands, Western Palearctic Region (Penney and Racek 1968).

Material examined. • CNPGG-2670, Cenote Sabak-Ha, Sacalum, Yucatan, Mexico (20.5801°N, 89.5881°W), 4 m deep, collected by Victor Eduardo Gómez Bretón, March 3rd 2024; • CNPGG-2671, Cenote Sabak-Ha, Sacalum, Yucatan, Mex-

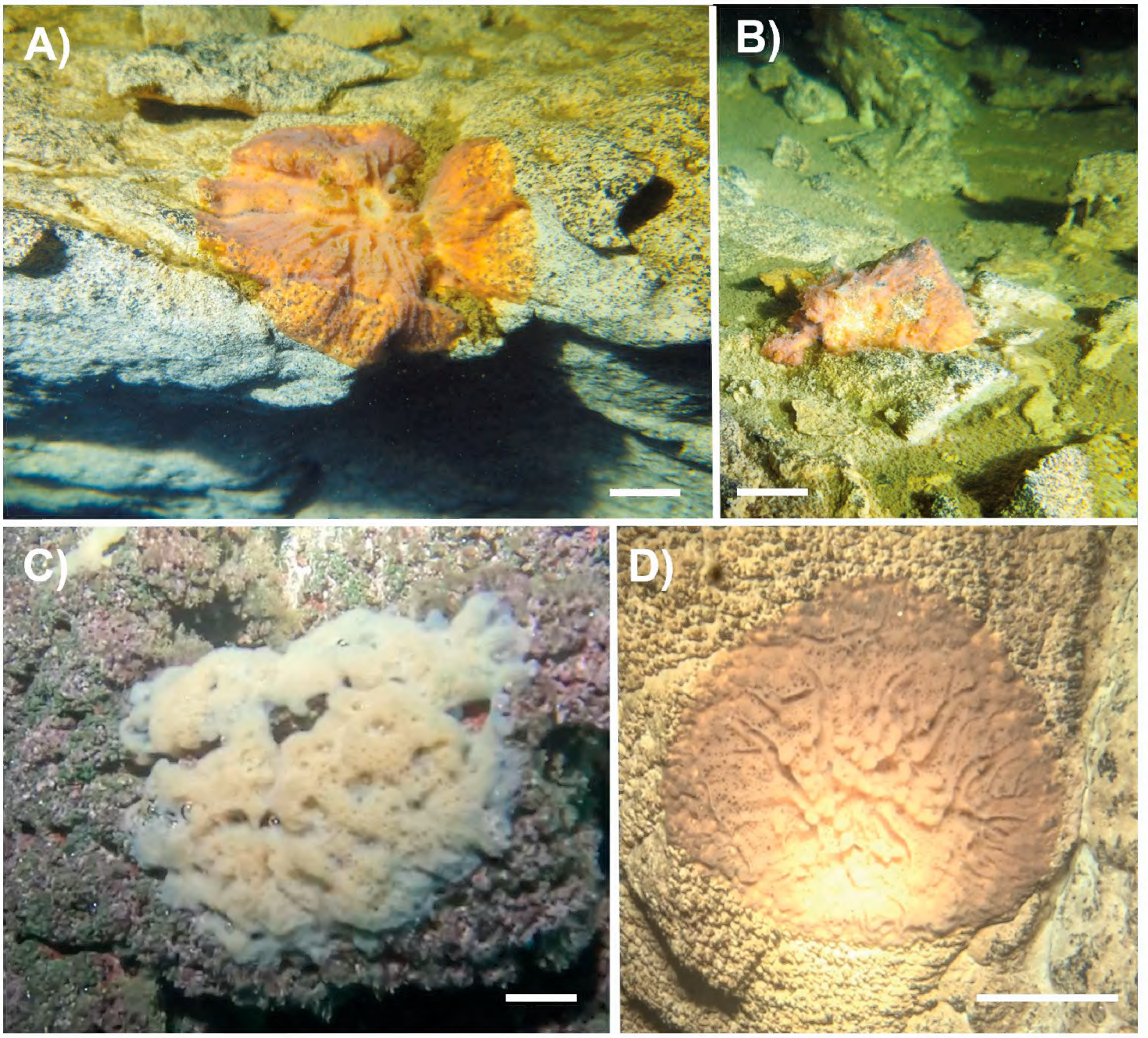


Figure 3. Morphological and size variations in *Ephydatia fluviatilis* from Sabak-Ha Cenote. **A** typically discoidal form, sponges in shaded areas at 8 m depth present a pink coloration and soft texture **B** growth form adapting to substrata instead of the typical discoidal form at 6 m depth **C** sponge CNPGG-2670 in lighted areas present yellow to green color and a slightly harder texture, collected at 4 m depth **D** sponge CNPGG-2671 collected at 11 m depth. Scale bars: 2 cm (**A**); 1 cm (**B**); 2 cm (**C**); 4 cm (**D**).

ico (20.5801°N, 89.5881°W), 11 m deep, collected by V. E. Gómez Bretón, March 3rd 2024; • CNPGG-2673, Cenote Suhem, Pixya, Yucatan, Mexico (20.6403°N, 89.4031°W), 5.3 m deep, collected by V. E. Gómez Bretón, March 29th 2025.

Distribution. Canada (Dawson 1875; Ricciardi and Reiswig 1993), China (Gee 1926; Zhang 1948), Himalaya (Annandale 1912), Estonia (Roovere et al. 2006), Germany (Linnaeus 1759), France (Topsent (1930)), Ireland (Stephens 1920), Italy (Cardone 2014), Mexico (Potts 1885a), Myanmar (Annandale 1918), Netherlands (Vorstman 1954; Van Soest 2025), Poland (Linnaeus 1759), South Africa (Kirkpatrick 1907; Manconi 2008), Sweden (Linnaeus 1759), United Kingdom (Evans and Montagnes 2019), United States (Lauer and Spacie 1996; Manconi and Pronzato 2016), Russia (Schletterer and Eggers 2006), Spain (Gost et al. 2023).

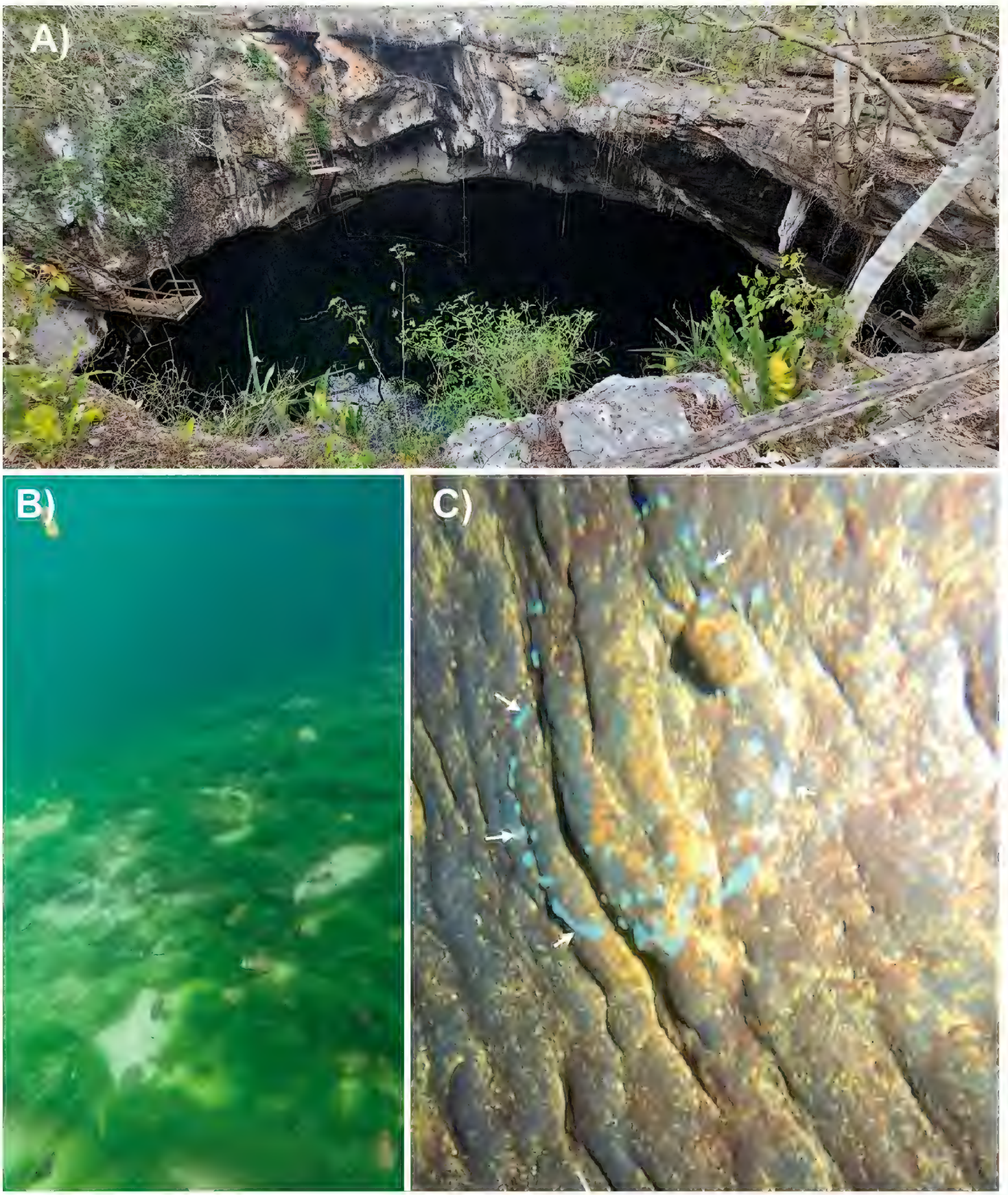


Figure 4. Suhem Cenote in Pixya, Yucatan. **A** external view of the cenote entrance **B** panoramic view of the cenote bottom at 3 m depth **C** detailed view of the rocky wall at 4 m depth showing sponge specimen locations (indicated by arrows delimiting a typical rounded area).

External description. In Sabak-Ha Cenote, the sponge exhibits a predominantly massive encrusting morphology, creating prominent mounded structures and frequently developing into distinctive discoidal formations. In contrast, specimens from the Suhem cenote present as diminutive, attenuated, and irregularly encrusting forms (Figs 3, 5). Smooth and porous texture. The consistency of the living sponge is fragile

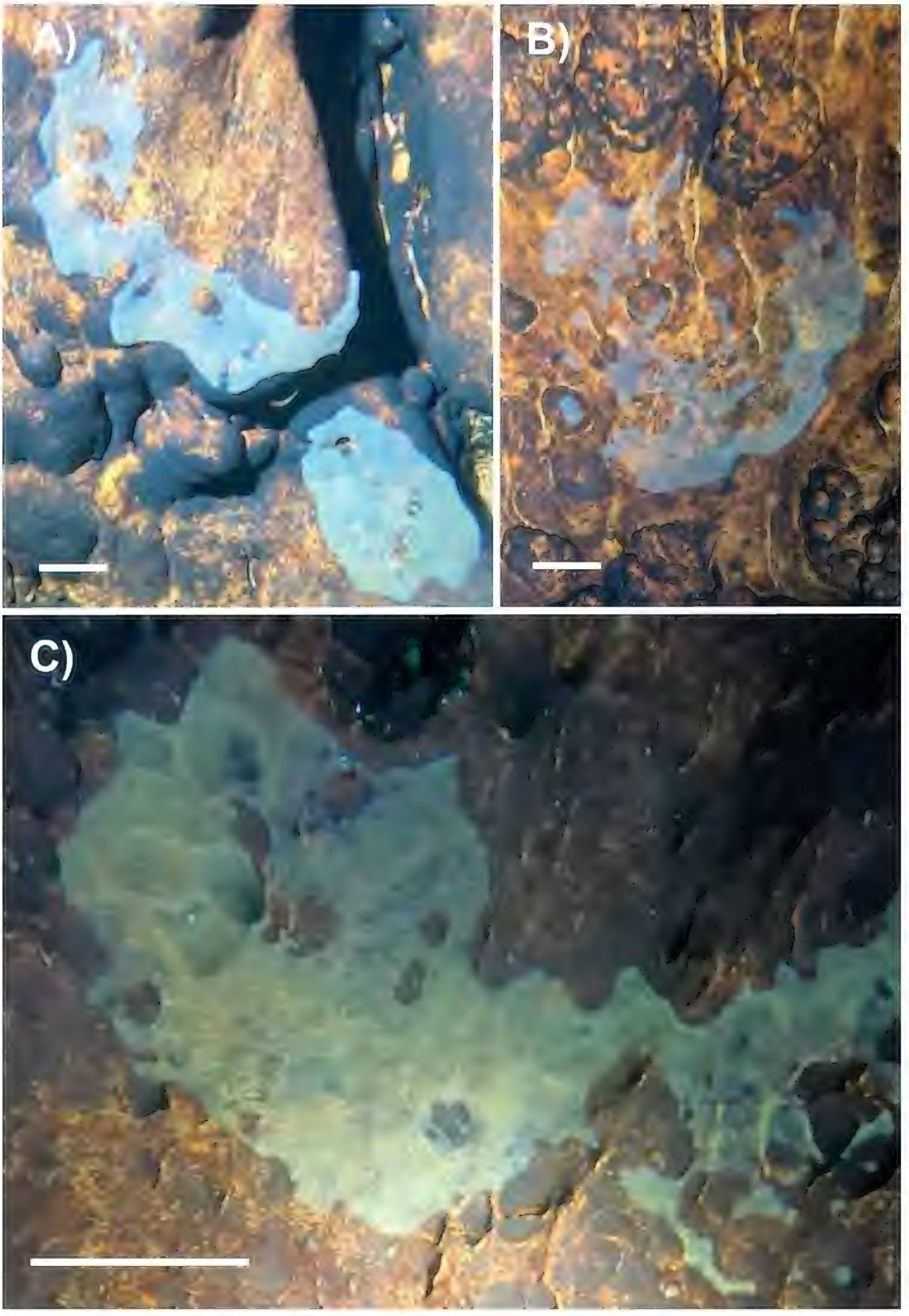


Figure 5. Morphological and size variations in *Ephydatia fluviatilis* from Suhem Cenote. Specimens collected at depths of 4 m (**A**), 4.7 m (**B**), and 5.3 m (**C**) **C** this photography belongs to the collected sponge CNPGG-2673. Scale bars: 1 cm (**A, B**); 4 cm (**C**).

and easily brittle upon contact. Oscula not visible to the naked eye. Exhibiting two colors *in vivo*, greenish yellow in the presence of light and dull pinkish in its absence (Fig. 3). Whitish color when preserved in alcohol.

Skeleton. Forming paucispicular tracts, composed of 2–5 intersecting oxea spicules producing regular mesh (Fig. 6D). The tracts form an alveolate structure, clearly forming rounded meshes. Spongin is generally scarce but present in most tracts.

Gemmules. Gemmules scarce and scattered, hemispherical measuring 200.56 (190.77–210.36) μm in diameter (Fig. 6A). Gemmules were observed exclusively in specimens from Suhem Cenote.

Spicules. Megascleres consist primarily of smooth oxeas that are slender and slightly curved at the midpoint, occurring in two distinct size categories. Oxeas I are the most common spicules in specimens from both cenotes, measuring 327.13 (243.28–373.53) μm × 15.15 (10.09–19.71) μm (Fig. 6B). Oxeas II are much less frequent than type I and are noticeably shorter and thinner, measuring 172.55 (147.27–199.68) μm × 4.7 (3.77–5.14) μm. These measurements were obtained from the total number of spicules measured in specimens CNPGG-2670 (Fig. 3C), CNPGG-2671 (Fig. 3D) from the Sabak-Ha Cenote and CNPGG-2673 (Fig. 5C) from the Suhem Cenote.

Additionally, smaller and less abundant spiny oxeas were observed; these are mostly straight and were found exclusively in specimen CNPGG-2670 from the Sabak-Ha Cenote. They measure 110.71 (88.92–132.51) μm × 10.13 (9.10–11.17) μm (Fig. 6C).

Gemmuloscleres consist of birotules with spined shafts and rotules of nearly identical morphology at both extremities (Figs 6F, 7), measuring 46.76 (32.66–72.96) μm × 7.04 (4.64–10.15) μm. The rotules have a diameter of 23.41 (19.18–31.74) μm (Fig. 6G). This size range was obtained from the total number of gemmulosclere birotules measured in all three collected specimens: CNPGG-2670 and CNPGG-2671 from the Sabak-Ha Cenote, and CNPGG-2673 from the Suhem Cenote. Detailed measurements for each specimen are provided in Table 1.

Molecular results. The molecular analyses using Bayesian and Maximum Likelihood methods yielded congruent topologies, revealing a well-supported monophyletic clade containing specimens together with *Ephydatia fluviatilis*, confirming species-level identification (Fig. 8). Sequences from both cenotes (Sabak-Ha and Suhem) were identical in nucleotide composition, and BLAST searches against GenBank confirmed assignment to Spongillida (Porifera), with our samples differing by less than 3% from reference sequences of *E. fluviatilis*. This clade clustered as sister to a monophyletic group formed by *Heterorotula* (Penney & Racek, 1968) and *Racekiela* (Bass & Volkmer-Ribeiro, 1984), with both clades sister to the main *Ephydatia* assemblage. The genera *Eunapius* (Gray, 1867) and *Spongilla* (Lamarck, 1816) were recovered as monophyletic, while *Trochospongilla* and *Radiospongilla* remained unresolved.

Remarks. The morphological and molecular results strongly support the assignment of the specimens to the genus *Ephydatia*. First, BLAST-based molecular analyses confirmed the identity of our specimens within the Spongillidae family, revealing that the samples present a divergence of less than 3% from *Ephydatia fluviatilis*. This low genetic divergence is common in the *E. fluviatilis* group and indicates a very close relation-

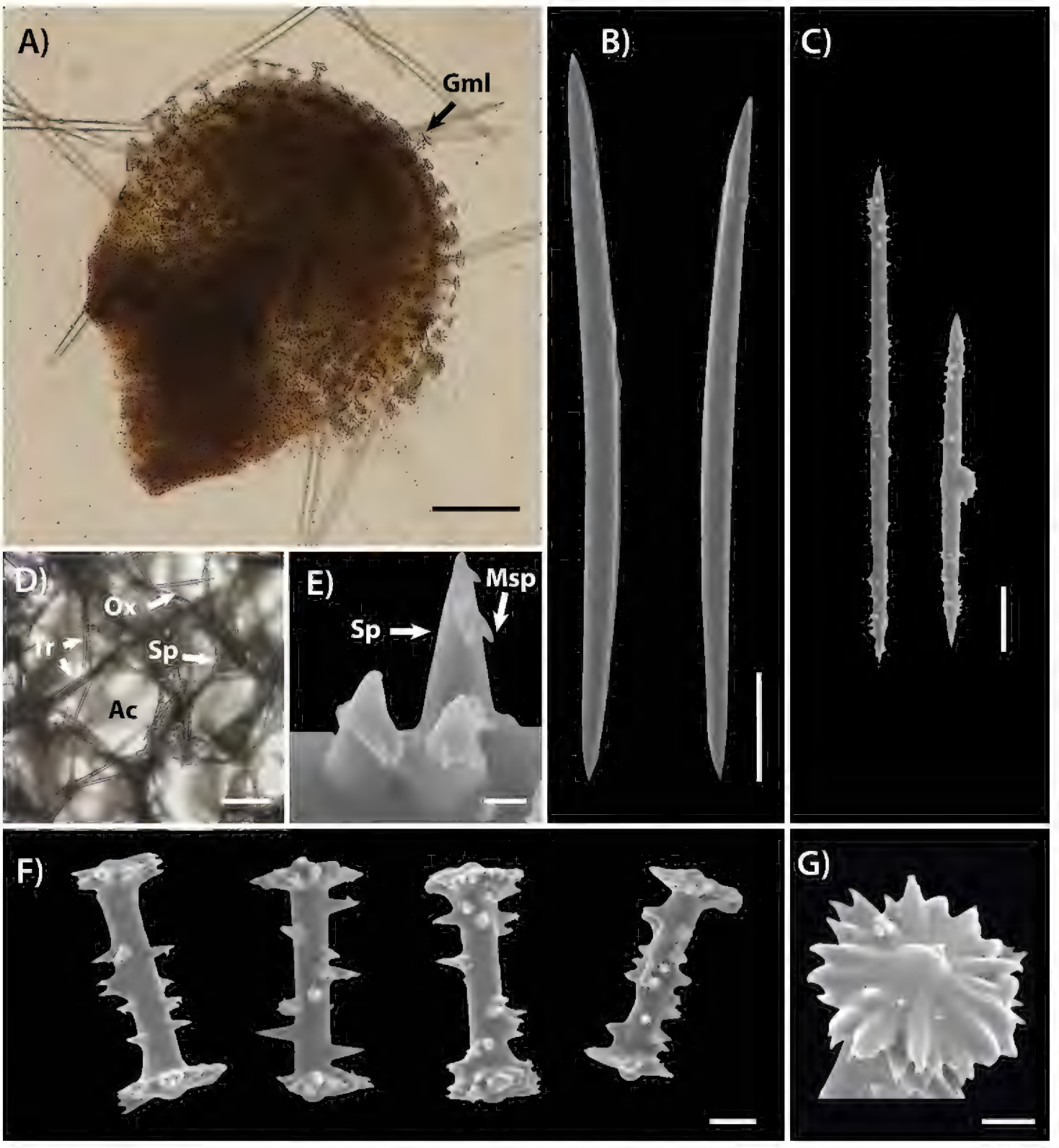


Figure 6. Gemmule, spicules and skeletal architecture of *Ephydatia fluviatilis* from Sabak-Ha and Suhem Cenotes. **A** gemmule with visible birotules (Gml) **B** smooth oxeas **C** spiny oxeas exclusively from Sabak-Ha Cenote sampled sponges **D** skeletal network tracts (Tr) composed of oxeas (Ox), spongin (Sp) and alveolate cavities (Ac) by LM **E** spines (Sp) and microspines (Msp) of the birotules shaft by SEM **F** birotules gemmuloscleres by SEM **G** detail of a rotule (top view) by SEM **A** taken from specimen CNPGG-2673 **B–E, G** taken from the specimen CNPGG-2670 **F** taken from specimens CNPGG-2670 and CNPGG-2671. Scale bars: 50 μm (**A, B**); 20 μm (**C**) 100 μm (**D**); 2 μm (**E**); 10 μm (**F, G**).

ship (Zhao et al. 2023). The molecular assignment was solidified through phylogenetic reconstruction, where the trees obtained by Maximum Likelihood (ML) and Bayesian Inference (BI) were congruent, demonstrating that the sponges from both cenotes consistently cluster together and form a robust clade alongside *Ephydatia fluviatilis*. In

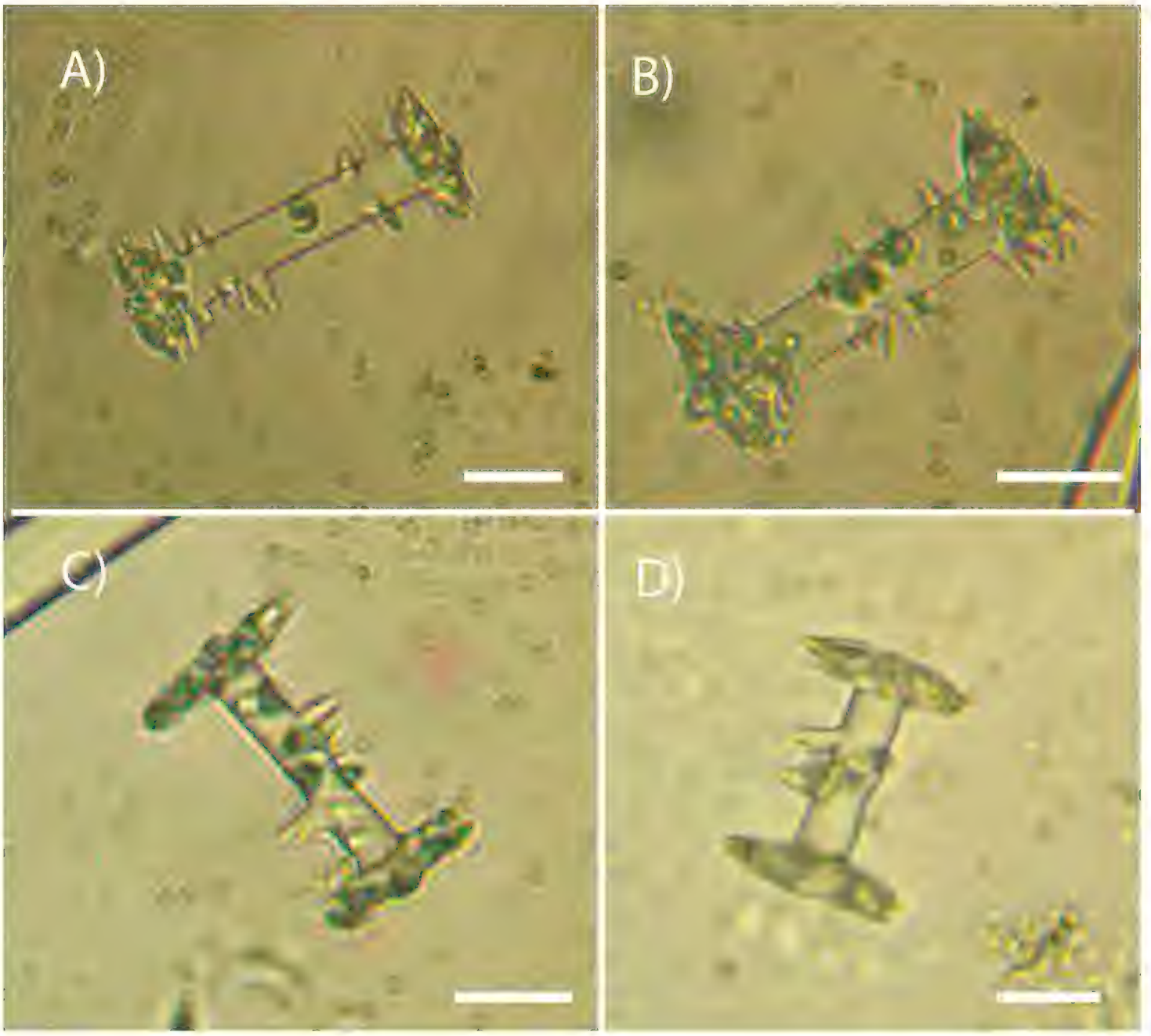


Figure 7. Comparison of birotules from sponges collected in Sabak-Ha Cenote (**A, B**) and Suhem Cenote (**C, D**). Birotule **A** corresponds to specimen CNPGG-2670 (Fig. 3C), and birotule **B** to specimen CNPGG-2671 (Fig. 3D). Birotule **C** corresponds to specimen CNPGG-2673 (Fig. 5C), while birotule **D** corresponds to a sponge from Suhem not deposited in the CNPGG collection (Fig. 5A). Scale bars: 10 μm.

this grouping, the *E. fluviatilis* group was positioned as a sister group to monophyletic clades composed of the genera *Heterorotula* (Penney & Racek, 1968) and *Racekiela cresciscrystae* (Gómez, Carballo, Cruz-Barraza & Camacho-Cancino, 2019). This particular topology is notable as it is consistent with previous studies on Spongillida phylogeny based on ITS sequences and underscores the taxonomic complexity of the group (Gómez et al. 2019; Itskovich et al. 2022; Zhao et al. 2023).

Regarding morphology, the genus *Ephydatia* is characterized by birotules with short shafts, rotules of equal diameter, and megasclere oxeas that may be either microspined or smooth (Manconi and Pronzato 2002), all features observed in our specimens. In addition, the morphology of the collected specimens is consistent with the bulbous and corrugated, flat encrustating and discoidal typical growth forms reported for *E. fluviatilis* (Penney and Racek 1968; Pronzato and Manconi 1995; Lopp et al. 2007).

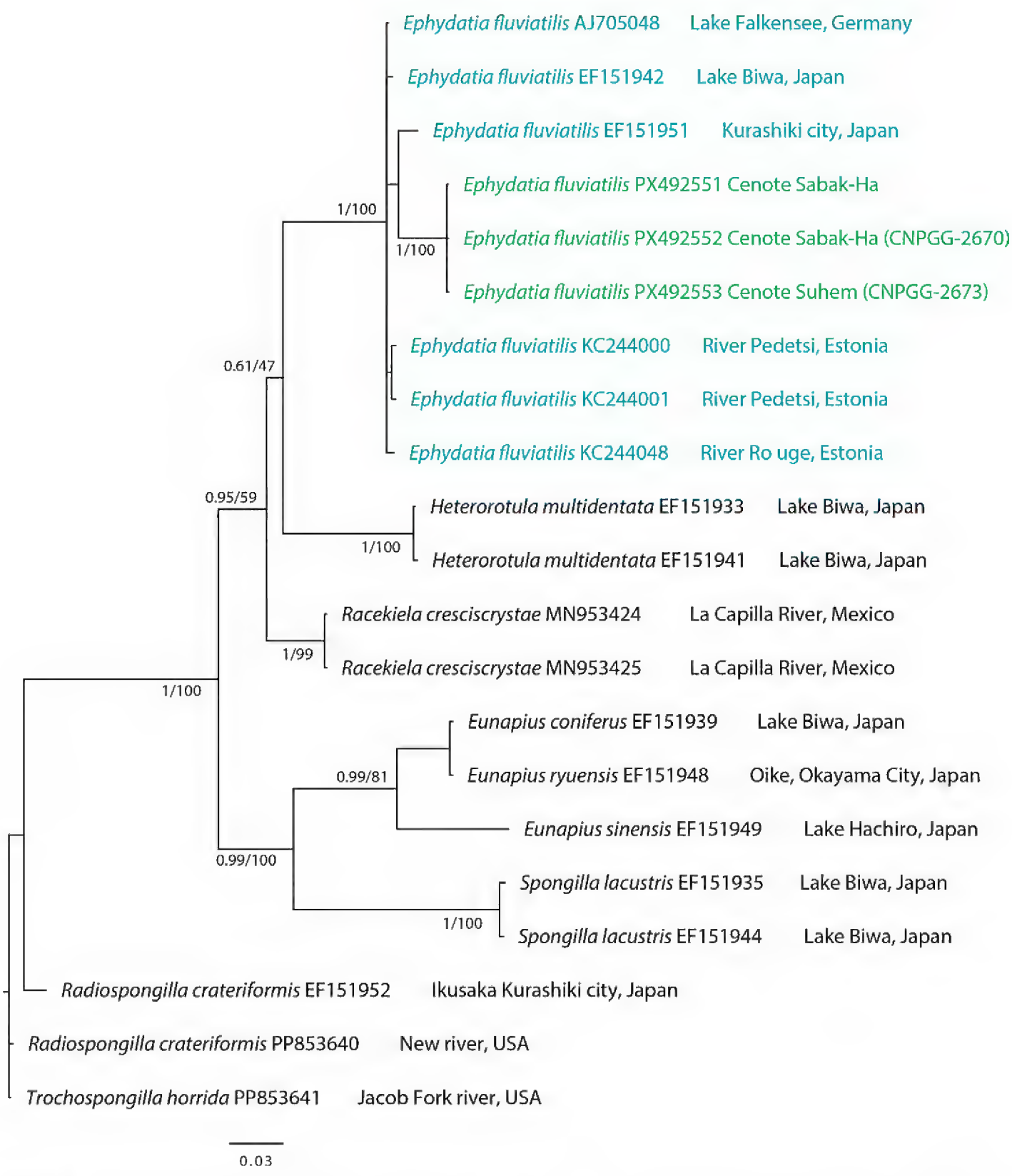

Ephydatia fluviatilis AJ705048 Lake Falkensee, Germany
Ephydatia fluviatilis EF151942 Lake Biwa, Japan
Ephydatia fluviatilis EF151951 Kurashiki city, Japan
Ephydatia fluviatilis PX492551 Cenote Sabak-Ha
Ephydatia fluviatilis PX492552 Cenote Sabak-Ha (CNPGG-2670)
Ephydatia fluviatilis PX492553 Cenote Suhem (CNPGG-2673)
Ephydatia fluviatilis KC244000 River Pedetsi, Estonia
Ephydatia fluviatilis KC244001 River Pedetsi, Estonia
Ephydatia fluviatilis KC244048 River Ro uge, Estonia
Heterorotula multidentata EF151933 Lake Biwa, Japan
Heterorotula multidentata EF151941 Lake Biwa, Japan
Racekiela cresciscrystae MN953424 La Capilla River, Mexico
Racekiela cresciscrystae MN953425 La Capilla River, Mexico
Eunapius coniferus EF151939 Lake Biwa, Japan
Eunapius ryuensis EF151948 Oike, Okayama City, Japan
Eunapius sinensis EF151949 Lake Hachiro, Japan
Spongilla lacustris EF151935 Lake Biwa, Japan
Spongilla lacustris EF151944 Lake Biwa, Japan
Radiospongilla crateriformis EF151952 Ikusaka Kurashiki city, Japan
Radiospongilla crateriformis PP853640 New river, USA
Trochospongilla horrida PP853641 Jacob Fork river, USA
1/100
1/100
0.61/47
0.95/59
1/100
1/99
1/100
0.99/81
0.99/100
1/100
0.03

Figure 8. Tree topology of rDNA region (18s-ITS1-5.8-ITS2-28s) based on comparisons of 813 bp from 21 freshwater sponge samples, obtained by Bayesian Inference (MrBayes) and Maximum Likelihood (PhyML). The number at each node represents the BI Posterior Probability, followed by ML Bootstrap Values. The GenBank sequence accession code was included after each species name. *Trochospongilla* PP853641 were used as outgroups. Taxon names of sequences obtained in this study are indicated in green.

In terms of spicule dimensions, the length of smooth oxeas I (243.28–373.53 μm) falls within the range reported in the original species description (210–400 μm) (Penney and Racek 1968). Notably, the specimen CNPGG-2670 (Fig. 3C) from Sabak-Ha also possessed uncommon, smaller, more heavily spined oxeas not previously documented in the literature. In addition, dimensions showed interpopulation variation, specimens from Sabak-Ha cenote exhibited longer birotules (44.65–72.96 μm) than

the typical range for *E. fluviatilis* (26–30 µm), while those from Suhem Cenote are more closely related to the expected range (32.66–43.15 µm) (Fig. 7) (Table 1). This variation suggests that site-specific environmental conditions within each cenote may influence spicule formation and growth, consistent with previous reports of this species sensitivity to changes in alkalinity and light intensity (Gost et al. 2023).

About the presence of spines in birotules, some studies have revealed them as response to the water chemistry, spine development has been linked to habitats with elevated chloride concentrations and high alkalinity (Poirrier et al. 1974), conditions characteristic of cenote ecosystems (Pérez-Ceballos et al. 2012; Cruz-Sánchez et al. 2018). These conditions allow the spination in birotules of *E. fluviatilis* in cenote ecosystems.

In North America, in addition to *E. fluviatilis*, the species *E. robusta* (Potts, 1888), *E. millsii* (Potts, 1888), *E. muelleri* (Lieberkühn, 1856), and *E. subtilis* (Weltner, 1895) have been reported (Carter 1885; Potts 1885b; Potts 1888; Weltner 1895; Smith 1918) (Fig. 9). However, descriptions of *E. robusta*, *E. millsii*, and *E. subtilis* remain

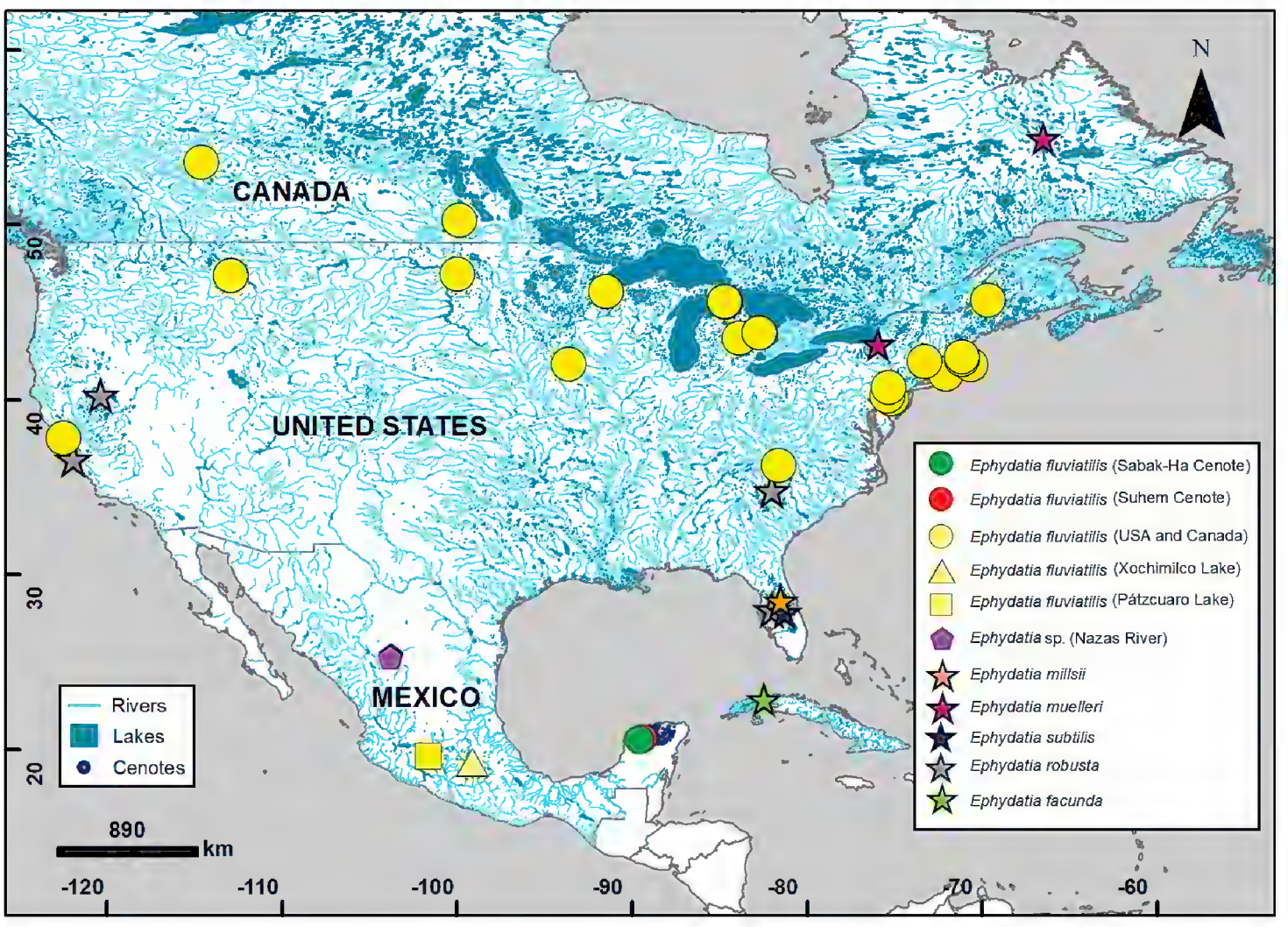


Figure 9. Distribution of six *Ephydatia* species across North America. Records of *E. fluviatilis* were obtained from the Global Biodiversity Information Facility (GBIF 2025a), with historical records in Mexico from Pátzcuaro and Xochimilco Lakes documented by Rioja (1942) and Penney and Racek (1968), respectively, and a recent record of *Ephydatia* sp. in Nazas River by Rodríguez-Ríos et al. (2024). Records of *E. millsii* and *E. robusta* were obtained from Potts (1888), with additional *E. robusta* records from Laubenfels (1932) and GBIF (2025b). Records of *E. muelleri* were obtained from Smith (1918) and Ricciardi and Reiswig (1993), records of *E. subtilis* were from Weltner (1895) and the record of *E. facunda* was from Manconi and Pronzato (2005).

outdated and have not been revised since their original descriptions. *E. robusta* was described as having large birotules with cylindrical or conical shafts bearing long spines (Potts 1888), while *E. millsii* presents symmetrical birotules with nearly cylindrical shafts bearing a single median spine (Potts 1888). The identity of *E. subtilis* remains uncertain (Weltner 1895; Harrison 1979). In contrast, *E. muelleri* is the only species with recent molecular and updated morphological data (Lopp et al. 2007; Kenny et al. 2020). *E. fluviatilis* is morphologically similar to *E. muelleri*, differing mainly in birotule shaft length and oxeas spination (Lopp et al. 2007), although their genetic relationship remains unclear (Zhao et al. 2023).

These limitations highlight gaps in species delimitation, as morphological differences are largely based on the presence or absence of spines on oxeas or birotule shafts. Given the wide distribution of *E. fluviatilis* across freshwater habitats in the Northern Hemisphere (Oscoz et al. 2009; Lucentini et al. 2013; Erpenbeck et al. 2020), integrative studies combining molecular and morphological data are required to improve species delimitation in this region and across the genus range (Fig. 9). Zhao et al. (2023) suggested that *E. fluviatilis* represents a species complex, with birotule shaft length being more informative than spine presence. Our morphological and molecular data support this view, indicating that shaft spines in Yucatan cenote populations reflect environmentally induced variation rather than species-level diagnostic characters.

Discussion

In Mexico, knowledge of the diversity and distribution of the freshwater sponge genus *Ephydatia* has remained outdated and underexplored. Old records report the species *E. fluviatilis* and *E. robusta* in Lake Xochimilco and Pátzcuaro (Zorrilla 1935; Old 1936a; Rioja 1942). Recently, the study by Rodríguez-Ríos et al. (2024) reported a sponge of the genus *Ephydatia* in Nazas River in Fernández Canyon, Lerdo, Durango; however, their study focused on insect larvae and the sponge species was not identified to species level. Therefore, our record of *E. fluviatilis* in cenote ecosystems extends the distribution range, updates the morphological description and integrates the first molecular data for this species in Mexico.

Earlier records are taxonomically problematic, Zorrilla (1935) and Old (1936a) merely mentioned the presence of *E. robusta* in Lake Xochimilco without providing formal descriptions, photographs, or illustrations to support identification. These authors variously identified specimens of the genus *Ephydatia* from this lake as *Ephydatia subdivisa* (Potts, 1888) (now recognized as a synonym of *E. robusta*), *E. viridis* (a taxonomically uncertain designation), and *E. crateriformis* (now reclassified as *Radiospongilla crateriformis*). Such inconsistencies underscore the considerable uncertainty that has historically characterized our knowledge of the genus *Ephydatia* in Mexico.

Recent taxonomic research examining freshwater sponge specimens collected during the last century has proven that historical identifications frequently require revision when subjected to contemporary molecular analysis and detailed morphological

examination of spicules and gemmules. This need for taxonomic reassessment has been clearly demonstrated in studies of the genera *Racekiela* (Bass & Volkmer-Ribeiro, 1998) and *Heteromeyenia* in Mexico (Carballo et al. 2017, 2021). Consequently, a comprehensive review and update of the reported occurrences of *Ephydatia* species is essential to clarify the actual diversity and distribution of this genus in Mexico.

Despite the potential taxonomic ambiguity in *Ephydatia* species in Mexico, our study updates the biodiversity records of cenote sponges in Yucatan. Prior to this research, *Ephydatia* had not been documented in these ecosystems. With the inclusion of our records and the previously reported *Spongilla cenota* and *Radiospongilla crateriformis* (Old 1936b; Penney and Racek 1968), the number of known cenote sponge species has increased to three, each from a different genus. Additionally, two new cenotes were added to existing records, resulting in six cenote ecosystems with freshwater sponge populations (Table 2). Given the high number of cenotes in Yucatan (POETY 2021), further undiscovered species are likely.

Both previous and current studies consistently recorded sponges in open-type cenotes with vertical walls (Hall 1936; Beddows et al. 2007), suggesting that these environments may favor sponge presence. These cenotes receive sufficient light to support primary productivity and associated microalgae (Godefroy et al. 2019). Light exposure may also explain the observed color variation, as greenish-yellow coloration in illuminated specimens is likely linked to photosynthetic symbionts, a phenomenon reported in marine and freshwater sponges (Lemloh et al. 2009; Giudice and Rizzo 2024) and in other *Ephydatia* species (Hustus et al. 2023).

Future studies should aim to verify the records of *S. cenota* and *R. crateriformis* documented by Old (1936b) in their original locations. These include Xanaba Cenote Grande, Xanaba Cenote, and Dzadz Aguada, which are currently referenced only by their near location from the archaeological site Chichen Itzá (Table 2), rendering their precise geographical positioning is uncertain. Specifically, the Xanaba cenotes lack entries in the existing cenote database (POETY 2021), while multiple cenotes named Dzadz Aguada are located in different regions from Old's (1936b) original description. This geographic ambiguity prevented us from accurately representing *R. crateriformis* locations on our cenote sponge map (Fig. 1). Instead, we provide an estimated reference point centered around Chichen Itzá, where these historical records might be situated.

Finally, this study emphasizes the importance of documenting and recording sponge populations in cenote ecosystems of Yucatan. With sponges frequently being overlooked in biodiversity inventories of cenotes, there has been a significant information gap for over 40 years regarding this taxonomic group (Cervantes-Martínez et al. 2018; Mejía-Ortiz and Yáñez 2024). As a result, numerous fundamental aspects of sponge ecology and biology within these ecosystems remain unknown, creating valuable opportunities for future research. These research opportunities include investigating the specific environmental conditions that determine sponge presence and absence in cenotes, as well as understanding their spatiotemporal distribution patterns throughout the cenotes network in Yucatan.

Research on freshwater sponges can provide important insights into water chemical conditions (Hill and Hill 2002), illustrate the evolutionary history of ecosystems (Erpenbeck et al. 2020), and offer valuable resources for biotechnological applications (Keller-Costa et al. 2014; Giudice and Rizzo 2024). Additionally, comprehensive sponge records will help complete the understanding of species diversity in cenotes, potentially supporting conservation efforts for these unique ecosystems. It is noteworthy that most of the cenotes where sponges have been reported are not located within the "Reserva Estatal Geohidrológica Anillo de Cenotes," a Natural Protected Area (known in Spanish as ANP) established in 2013 (SDS 2013). Given this situation, it is imperative to intensify research efforts focused on understanding sponge populations together with their associated invertebrates, microalgae and microbiome in Yucatan's cenote ecosystems.

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